An imperfect G2M checkpoint contributes to chromosome instability following irradiation of S and G2 phase cells

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Extra Views:

An imperfect G2/M checkpoint contributes to chromosome instability following irradiation of S and G2 phase cells

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Abstract

DNA double strand break (DSB) repair and checkpoint control represent two major mechanisms that function to reduce chromosomal instability following ionising irradiation (IR). Ataxia telangiectasia (A-T) cells have long been known to have defective checkpoint responses. Recent studies have shown that they also have a DSB repair defect following IR raising the issue of how ATM's repair and checkpoint functions interplay to maintain chromosomal stability. A-T and Artemis cells manifest an identical and epistatic repair defect throughout the cell cycle demonstrating that ATM's major repair defect following IR represents Artemis-dependent end-processing. Artemis cells show efficient G2/M checkpoint induction and a prolonged arrest relative to normal cells. Following irradiation of G2 cells, this checkpoint is dependent on ATM and A-T cells fail to show checkpoint arrest. In contrast, cells irradiated during S phase initiate a G2/M checkpoint which is independent of ATM and, significantly, both Artemis and A-T cells show a prolonged arrest at the G2/M checkpoint likely reflecting their repair defect. Strikingly, the G2/M checkpoint is released before the completion of repair when approximately 10-20 DSBs remain both for S phase and G2 phase irradiated cells. This defined sensitivity level of the G2/M checkpoint explains the prolonged arrest in repair-deficient relative to normal cells and provides a conceptual framework for the co-operative phenotype between checkpoint and repair functions in maintaining chromosomal stability.

Cells initiate DNA damage checkpoints after genotoxic stress to provide time for the repair of DNA damage. Whereas the G1/S and the intra-S checkpoints prevent cells from unfaithful replication of their genomes, the G2/M checkpoint is initiated to allow repair of DNA damage prior to mitosis. DNA repair and checkpoint induction are therefore closely interlinked to guarantee genomic stability and prevent cells undergoing malignant transformation.[1-5] Key players in the signalling events that induce cell cycle arrest and DNA repair after damage are members of the PIKK3-like kinase family, namely ataxia telangiectasia mutated (ATM) and ataxia telangiectasia mutated and rad3 related (ATR). Whereas ATR is believed to respond to damage after UV and breaks resulting from DNA replication, ATM is the prominent signalling molecule involved in the response to DNA double strand breaks (DSBs) induced by ionizing radiation (IR).[6-9] The central position of ATM in the maintenance of genomic stability becomes apparent by its involvement in checkpoint regulation at the G1/S, intra-S, and G2/M transition. Being one of the earliest known responders to DNA-damage, ATM signals to a multitude of proteins involved in DNA repair, checkpoint control and apoptosis.[10-12] The genomic disorder Ataxia telangiectasia (A-T) conferred by a defect in the gene, *ATM*, is characterized by neurodegeneration causing progressive ataxia, telangiectasia, a high cancer predisposition, and extraordinary radiation sensitivity. Radiation sensitivity and cancer predisposition have generally been attributed to the checkpoint defects of A-T cells.[13-16] More recently, it has been demonstrated that ATM also participates directly in the repair of a subset of IR-induced DSBs that contributes to radiation sensitivity of A-T cells.[17-20] DSBs are a severe genotoxic lesion because if unrepaired they compromise genomic integrity and if misrepaired they can cause genomic instability. In mammalian cells, non-homologous end joining (NHEJ) is the main DSB repair pathway. In S and G2 phase, homologous recombination (HR) constitutes a second pathway to repair DSBs.[21-24] In confluent cells, the ATM-dependent repair pathway depends on functional DNA-PKcs, a classical component of NHEJ. Therefore, ATM-dependent DSB repair was classified as a sub-pathway of NHEJ. In

addition to ATM, the repair pathway is also dependent on the nuclease Artemis and several proteins known to form IR-induced repair foci, such as γH2AX and 53BP1. The fact that Artemis is a nuclease, that the ATM- and Artemis-dependent repair pathway constitutes the slow component of DSB repair, and that DNA damaging treatments that induce DSBs of varying complexity produce differing repair kinetics in A-T and Artemis cells led to the hypothesis that complex DSBs are the main substrate of this sub-pathway.[18-20,25] In a recent paper by Deckbar et al.[26] the repair studies were extended to proliferating cells, and it was shown that an ATM- and Artemis-dependent repair pathway of similar magnitude to that observed in G0/G1 also operates in G2 phase cells.

We predicted that a DSB repair defect in G2 phase immediately before cell division might substantially contribute to chromosome instability by enhancing the appearance of chromosomal aberrations. We, therefore, aimed to evaluate the relative contribution of ATM's repair and checkpoint functions to the maintenance of genomic stability. Classical chromosomal analysis of metaphase spreads revealed that repair-deficient Artemis cells have elevated levels of chromosome breaks per mitotic cell relative to normal wild-type (WT) cells. Checkpoint abrogation by chemical inhibition of Chk1/2 also increases the level of chromosome breaks per mitotic cell relative to WT cells but a combined checkpoint and repair defect (represented by A-T cells or by Artemis cells treated with a Chk1/2 inhibitor) is more severe than the sum of each defect alone. However, classical metaphase break analysis only provides an estimate for the average number of breaks in single cells that overcome the checkpoint at a specific time after irradiation, but fails to reveal the behaviour of the whole cell population. As a novel concept for analysing mitotic breakage, we, therefore, considered not only the number of mitotic breaks per cell but additionally the percentage of cells entering mitosis at each time point. To study checkpoint induction and release following IR in G2, we used BrdU labelling to identify G2 phase cells. Following BrdU pulse labelling in the absence of irradiation, the BrdU-positive cells progress into G2 within about 4 h and by 12 h have

exited G2 (Figure 1A, dotted lines). Hence, we irradiated BrdU-labelled cells with 1 Gy at 4 h post labelling when BrdU-positive cells represent those in G2 (Figure 1A). Time course analysis by two-dimensional FACS revealed that checkpoint-deficient A-T cells continuously enter mitosis whilst WT cells initiate a checkpoint upon irradiation that is sustained for at least 4 h and then rapidly leave G2 and progress into G1. Artemis cells, unlike A-T cells, are able to induce a G2/M checkpoint after irradiation in G2 phase. Checkpoint release in Artemis cells occurs later than in WT cells, which likely reflects their repair defect (Figure 1A). By 12 h post irradiation, most cells have reached the subsequent G1 phase. The combination of these FACS data with the results from the classical metaphase break analysis described above reveals that the majority of A-T cells enter mitosis at early times post irradiation with a multitude of chromosomal breaks. Most likely, this significantly contributes to their high genomic instability. In contrast, in checkpoint-proficient cells only a small number of cells fail to arrest at the G2/M checkpoint. Although these cells have an elevated number of breaks, they have little impact on the genomic stability of the irradiated population because they represent such a low percentage. In contrast, at later times when the majority of checkpoint-proficient cells are released from the G2/M checkpoint, most breaks have been repaired. Yet, checkpoint-proficient cells still contain on average 1-2 mitotic breaks per cell, which is >10-fold above the background number of chromosome breaks. These data are substantiated by the analysis of chromosomal breaks after premature chromosome condensation (PCC) in G2, and by comparison with γH2AX foci kinetics of the respective cell lines. Indeed, G2/M checkpoint-proficient cells are released when they still contain about 3-4 PCC breaks per cell, or 10-20 γH2AX foci. Therefore, checkpoint release occurs before the completion of repair and represents a major mechanism by which chromosome aberrations are generated. Remarkably, the total number of chromosome breaks is similar in WT and Artemis cells although release of the checkpoint occurs at later times in the repair-deficient cells.[26] These stunning results led to the hypothesis that a threshold DSB level exists below which the G2/M

checkpoint is abrogated. As shown in Figure 1B, G2 phase cells exhibit an average γH2AX level of about 30 foci at a time point before the checkpoint is released and a mean foci number of about 15 after the majority of cells are released from the checkpoint. There appears to be little selection for cells entering mitosis as similar foci numbers were counted in mitotic cells and G2 phase cells at identical times.[21,26,27] G2 phase A-T cells show the same γH2AX repair kinetics as Artemis cells but due to their G2/M checkpoint defect most cells progress into G1 at early times with a higher level of unrepaired DSBs.

In the study of Deckbar et al.[26] only cells irradiated in G2 phase were analysed. However, Xu et al.[28] described two molecularly distinct G2/M checkpoints. Cells irradiated in G2 phase show checkpoint induction that is dependent on functional ATM. In contrast, cells irradiated in S phase execute a G2/M checkpoint that is independent of ATM, most likely initiated by the activity of ATR. This prompted us to ask whether cells irradiated in S phase are also released into mitosis before completion of repair and whether the threshold level observed in G2 irradiated cells applies to S phase irradiated cells. In analogy to the G2 studies, primary WT, A-T and Artemis fibroblasts were pulse labelled with BrdU and immediately after labelling were irradiated with 1 Gy. Hence, the BrdU-labelled cells represent those in S phase at the time of irradiation. Progression of cells through the cell cycle was again analysed by FACS at various time points after irradiation. When compared to unirradiated cells, 1 Gy IR caused only a slight slowing-down of S phase progression in the first 4 h in all three cell lines. However, BrdU-positive cells accumulate in G2 over the next 4 h and the highest proportion of BrdU-positive G2 cells is observed 8 h after IR (Figure 1C). This is in contrast to G2 irradiated cells which show the maximum checkpoint arrest in the first 4 h post IR. Significantly, A-T and Artemis cells both show a prolonged arrest at the G2/M border, consistent with the G2 checkpoint for cells irradiated in S phase being ATM independent.[28] Release from G2 into G1 of these cells irradiated in S phase extends over 16 h. γH2AX analysis of the S phase irradiated cells which had progressed into G2 revealed a foci

level >20 at a time point before the checkpoint is released and mean foci numbers <20 after the majority of cells are released from the checkpoint (Figure 1D). This reflects the situation observed in G2 irradiated cells with the only difference that A-T cells induce the checkpoint and are released at the same time as Artemis cells, i.e. later than WT cells. The similar behaviour of Artemis and A-T cells also indirectly suggests that both factors are involved in the repair of DSBs induced during S phase. Foci levels of about 25 at 8 h post IR with 1 Gy in S phase cells are surprising considering the fact that IR should induce less DSBs in S phase than in G2 phase cells due to their lower DNA content. It is likely that replication past single strand breaks or base damage may cause additional DSBs if replication occurs before repair. It is also possible that DSBs become converted to a more unrepairable form if associated with a collapsed replication fork. Indeed, preliminary repair kinetics of S phase cells obtained in our laboratory suggest that during S phase no observable DSB repair occurs up to 4 h after irradiation when many cells had actually reached late S/G2 phase, consistent with the notion that additional DSBs are generated or DSB repair is impaired. Collectively, the data provide strong evidence that the release from the G2/M checkpoint is dependent on the residual number of unrepaired DSBs (about 20 γH2AX foci in the primary human cells studied here), irrespective of whether the cells were irradiated in S or G2 phase.

The hypothesis that a threshold level of unrepaired DSBs serves as a signal for release from the G2/M checkpoint implies that the time until cell cycle progression is resumed depends on the initial number of DSBs and hence on the applied dose. To test the dose dependence of checkpoint release, BrdU-labelled WT cells were irradiated in S phase with different doses of IR and the checkpoint arrest was monitored by FACS analysis. As expected, the length of the checkpoint arrest increased with dose (Figure 2A and Deckbar et al.[26]). At a dose of 9 Gy the checkpoint was sustained for more than 16 h. We also tested the dose dependence of checkpoint release in Artemis cells, to examine the impact of their repair defect. We predicted that higher doses of IR should eventually result in levels of unrepairable

breaks that inflict a permanent arrest in G2. Indeed, following 1 Gy IR cell cycle progression of Artemis cells was delayed when compared to identically treated WT cells but release did occur (e.g.: cell cycle delay in Artemis cells after 1 Gy is comparable to that of WT cells after 3 Gy) (Figure 2B). However, when BrdU-labelled Artemis cells were irradiated with 6 Gy, the cells remained in G2 for the entire observation period. Compared to the cell cycle behaviour of WT cells, this resembled the curve progression of WT cells exposed to 9 Gy irradiation. In a recent study by Syljuasen et al.[27] cells irradiated with a high dose of IR left G2 with multiple DSBs after long incubation times. They ascribed the G2/M checkpoint release to a process termed *checkpoint adaptation*. This phenomenon is best known in yeast and describes the fact that after a prolonged arrest in G2, cells are released with high levels of DNA damage.[29,30] However, the results of Syljuasen and co-workers are also in accordance with a threshold level of checkpoint release as their conclusions were based on a single high dose. To closely monitor checkpoint behaviour at longer times post irradiation with high doses, studies were conducted with 6 Gy and 9 Gy irradiated WT and Artemis cells. Although WT cells at these doses remain in G2 for a prolonged period of time relative to lower doses, they are eventually released from the checkpoint. In contrast, the majority of Artemis cells is retained in G2 up to 4 days after IR (Figure 2C). When γH2AX foci were counted in Artemis cells irradiated in S phase with 9 Gy and retained in G2 for up to 4 days, we observed ~50 foci (Figure 2D), a level that clearly exceeds the checkpoint threshold of 10-20 foci. WT cells likely repair DSBs even after 9 Gy irradiation. It is interesting to note that a level of ~50 unrejoined DSBs in Artemis cells irradiated during S phase with 9 Gy is consistent with an Artemis-dependent fraction of about 10% (based on about 50-60 induced DSBs per Gy per S/G2 cell), similar to that observed previously for G0 cells[18] and recently for G2 cells[26]. Thus, Artemis cells (and A-T cells) appear to exhibit a similar repair defect in all cell cycle phases.

The unexpected finding of a G2/M checkpoint threshold of ~10-20 DSBs raises the question of why this checkpoint should be released prior to the completion of repair. It has

been demonstrated by Deckbar et al.[26] that not only checkpoint release but also full induction of the G2/M arrest is dependent on the level of damage being above 10-20 γH2AX foci. It is, therefore, likely that both processes are governed by overlapping signalling pathways. As induction of the checkpoint is clearly dependent on the signalling activities of ATM and/or ATR, downstream targets of the kinases could be involved in these particular responses.[7,8,31,32] H2AX and 53BP1 are mediators of the ATM and ATR checkpoint signals, and checkpoint defects have been observed at low but not high doses of IR in H2AX- and 53BP1-deficient cells.[33] Furthermore, both signalling pathways result in the inactivation of Cdc25C and the following suppression of Cdk1/CyclinB. In order to arrest cells, Cdc25C has to be effectively removed from the nucleus by phosphorylation.[34] It is credible to speculate that below a certain level of DSBs the transmission of the damage signal is not strong enough to completely deplete the nucleus of Cdc25C. Consequently, Cdk1/CyclinB remains or becomes activated and mitosis can progress. Another possibility is that cell cycle promoting pathways such as activating phosphorylations on Cdks outweigh the checkpoint signal below a certain damage level. This has major implications for irradiation with low doses that induce less DSBs than would be necessary to fully induce the arrest (below about 0.3 Gy). In fact, the phenomenon of low-dose hypersensitivity, an extreme radiation sensitivity of proliferating checkpoint-proficient cells at low doses that results from ineffective cell cycle arrest of G2 phase cells,[35] can be explained by a threshold concept.

The data presented here and in Deckbar et al.[26] show that the Artemis/ATM component of DSB repair operates throughout the mammalian cell cycle. This component affects a subset of IR-induced DSBs, which we argued previously represent those which require end-processing.[18,19] Since our previous work established that Artemis-dependent DSB repair in non-cycling G0 cells is epistatic to DNA-PKcs and DNA ligase IV, we suggested that Artemis functions to process the break ends prior to ligation by NHEJ, a finding consistent with the notion that NHEJ is the predominant DSB repair process in the G1/G0

phase of the cell cycle. During the S and G2 phases of the cell cycle, however, additional DSB repair pathways, most notably HR, may also function. One explanation for the similar level of Artemis/ATM-dependent DSBs in all cell cycle phases is that Artemis has a role in DSB repair processes other than NHEJ. Our findings could also indicate that the majority of DSBs by IR is repaired by NHEJ in all cell cycle phases. In support of this, we have previously shown that NHEJ operates throughout the cell cycle[21] and more recently observed that DNA ligase IV- and Ku80-deficient MEFs have a similar, major DSB repair defect in G2 as in G1 (data not shown). In any case, the observation that Artemis-dependent end-processing is important in all cell cycle phases supports the notion that DSB repair by HR cannot overcome the need for Artemis. The similar repair defect for Artemis and A-T cells throughout the cell cycle further suggests that ATR cannot activate Artemis to any significant degree in A-T cells and that ATM's major repair defect represents Artemis-dependent end-processing.

In conclusion, our studies provide strong evidence that G2/M checkpoint release following IR depends upon the level of residual DSBs and that a permanent arrest in G2 is only sustained when the damage level remains above the threshold. Significantly, it is not crucial whether the damage is induced in G2 phase cells or in S phase cells that subsequently progress into G2. During S phase, replication dependent as well as directly induced DSBs contribute to checkpoint signalling, and therefore checkpoints evoked by both, ATM and ATR, appear to have similar threshold levels, i.e. sensitivities (Figure 3). Our initial studies aimed at investigating the fate of the G2 irradiated cells indicate that they can pass through mitosis with multiple breaks and reach G1. Whether the lesions can then be repaired in repair-proficient cells, or whether they become permanently arrested in G1, has now to be elucidated.

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Figure Legends

Figure 1. G2/M checkpoint release after IR in G2 and S is determined by a sensitivity threshold of 10-20 DSBs.

(A) FACS analysis of BrdU-labelled primary human WT, Artemis, and A-T fibroblasts after IR in G2. Cells were pulse-labelled with BrdU for 1 h. 4 h after labelling (when the majority of the BrdU-labelled cells had progressed into G2), cells were irradiated with 1 Gy, fixed immeditaly or 4, 8 or 12 h post IR, and then stained with BrdU antibody and propidium iodide for FACS analysis. The percentage of BrdU-positive cells in G2 was assessed. Dotted lines represent the percentage of BrdU-positive cells in G2 without irradiation. Error bars indicate the SEM (Figure modified from Deckbar et al.[26]).

(B) γH2AX foci analysis of BrdU-labelled primary human WT, Artemis, and A-T fibroblasts after IR in G2. Cells were grown on cover slips and labelled with BrdU for 1 h. 4 h after labelling, cells were irradiated with 1 Gy and then fixed 4 h or 8 h later for immunofluorescence analysis (i.e. at 8 h and 12 h post labelling). To assess the number of γH2AX foci specifically in BrdU-positive G2 cells, cells were triple-stained against BrdU, γH2AX and CENP-F which can be used as a G2 phase marker (Deckbar et al.[26]). γH2AX foci were counted in cells that were double-positive for BrdU and CENP-F. Error bars represent the SEM.

(C) FACS analysis of BrdU-labelled primary human WT, Artemis, and A-T fibroblasts after IR in S. Cells were pulse-labelled with BrdU for 1 h and immediately irradiated with 1 Gy. 0, 4, 8, 12 and 16 h post IR, cells were fixed and stained with BrdU antibody and propidium iodide for FACS analysis. The percentage of BrdU-positive G2 cells was assessed. Dotted lines represent the percentage of BrdU-positive cells in G2 without irradiation. Error bars indicate the SEM.

(D) γH2AX foci analysis of BrdU-labelled primary human WT, Artemis, and A-T fibroblasts after IR in S. Cells were grown on cover slips, labelled with BrdU for 1 h and irradiated with 1 Gy immediately after labelling. At 8 h or 12 h post irradiation (i.e. at 8 h and 12 h post labelling) cells were fixed and stained for immunofluorescence analysis as in (B). γH2AX foci were counted in cells that were double-positive for BrdU and CENP-F. Error bars represent the SEM.

Figure 2: Dose dependence of G2/M checkpoint release following IR.

(A) G2/M checkpoint release after IR in primary human WT fibroblasts (MRC-5) was measured by FACS analysis. Cells were pulse-labelled with BrdU for 1 h and irradiated with 0, 1, 3, 6 or 9 Gy immediately after labelling. At 1, 4, 8, 10, 12 and 16 h post irradiation, cells were fixed and stained with BrdU antibody and propidium iodide. The percentage of the BrdU-positive cells in G2 was assessed. Error bars represent the SEM.

(B) G2/M checkpoint release after IR in primary human Artemis fibroblasts (F01-240) was measured by FACS analysis. Cells were treated as in (A), fixed at 1, 4, 8, 12 and 16 h post IR, and subsequently stained with BrdU antibody and propidium iodide. The percentage of the BrdU-positive cells in G2 was assessed. Error bars represent the SEM.

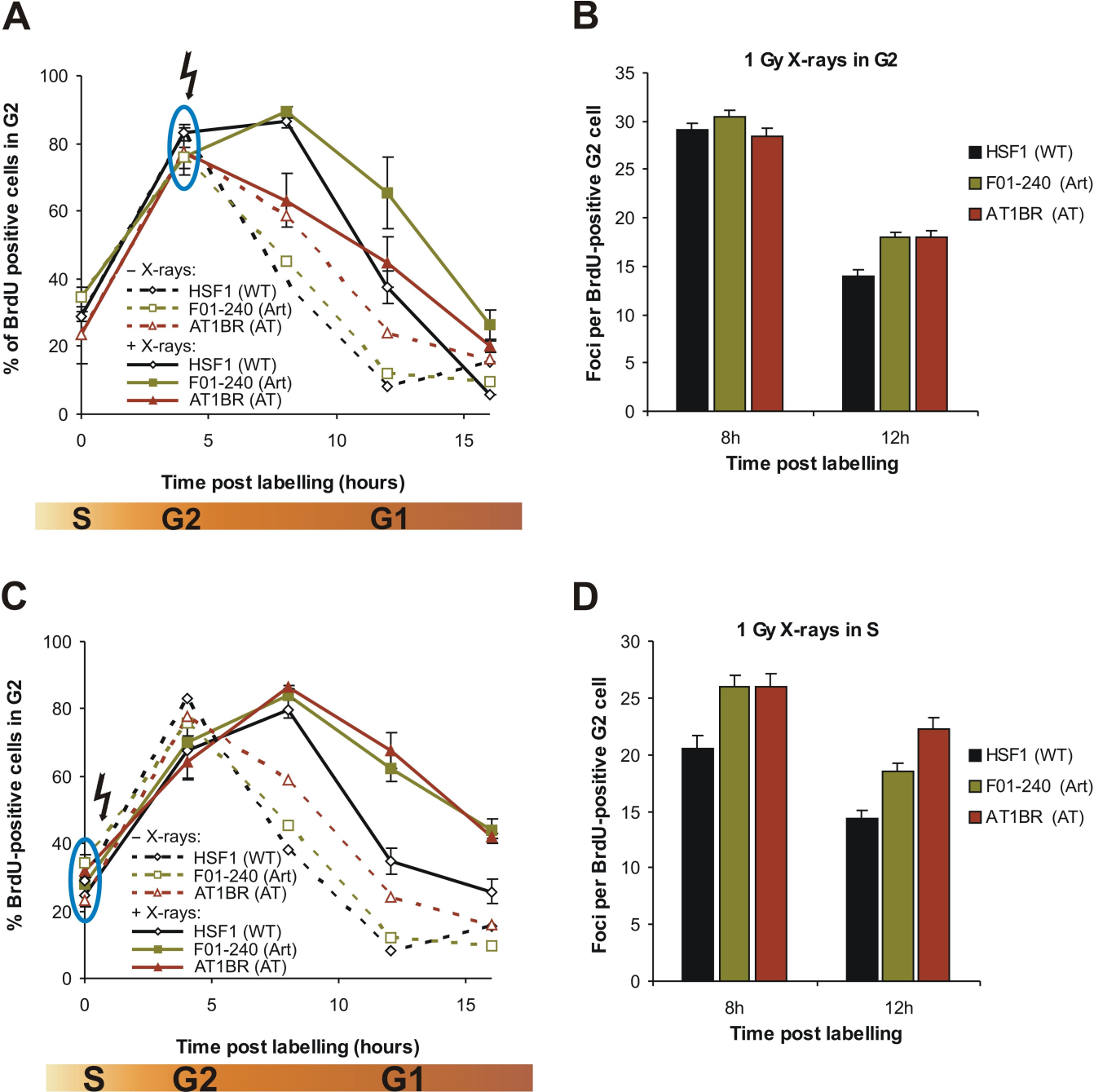
(C) G2/M checkpoint release after 6 Gy and 9 Gy in primary human WT and Artemis fibroblasts was monitored up to 4 days. Cells were BrdU pulse-labelled for 1 h and irradiated immediately after labelling with 6 Gy or 9 Gy. At 0, 8, 24, 48, 72 and 96 h post IR, cells were fixed and stained for FACS analysis. The percentage of the BrdU-positive cells in G2 was assessed.

(D) γH2AX foci analysis in primary human Artemis fibroblasts after 9 Gy irradiation was conducted up to 4 days post IR. Cells were grown on cover slips, pulse-labelled with BrdU for 1 h and irradiated with 9 Gy. At 24, 48, 72 and 96 h post irradiation, cells were

fixed for immunofluorescence analysis. After γH2AX/BrdU double staining, γH2AX foci numbers were assessed in the BrdU-positive cells. Error bars represent the SEM.

Figure 3: G2/M checkpoint response after irradiation in S or G2.

IR in S phase causes SSBs and DSBs that can result in extended regions of single-stranded DNA and new DSBs when the lesions encounter a replication fork before repair has occurred. Thus, ATR as well as ATM signalling can lead to the induction of a G2/M checkpoint. Hence, S phase irradiated A-T cells show proficient G2/M checkpoint induction. IR in G2 induces DSBs that result in ATM-dependent G2/M checkpoint arrest. Although strand resection occurs which can activate ATR this is dependent on ATM. Hence, G2 phase irradiated A-T cells fail to show G2/M checkpoint arrest. Strikingly, both S phase and G2 phase irradiated cells are released from G2/M checkpoint arrest into mitosis when they still harbour ~10-20 DSBs.


A
% of BrdU positive cells in G2
100
80
60
40
20
0
0
5
10
15
Time post labelling (hours)
– X-rays:
HSF1 (WT)
F01-240 (Art)
AT1BR (AT)
+ X-rays:
HSF1 (WT)
F01-240 (Art)
AT1BR (AT)
S
G2
G1
B
1 Gy X-rays in G2
Foci per BrdU-positive G2 cell
35
30
25
20
15
10
5
0
8h
12h
Time post labelling
HSF1 (WT)
F01-240 (Art)
AT1BR (AT)
C
% BrdU-positive cells in G2
100
80
60
40
20
0
0
5
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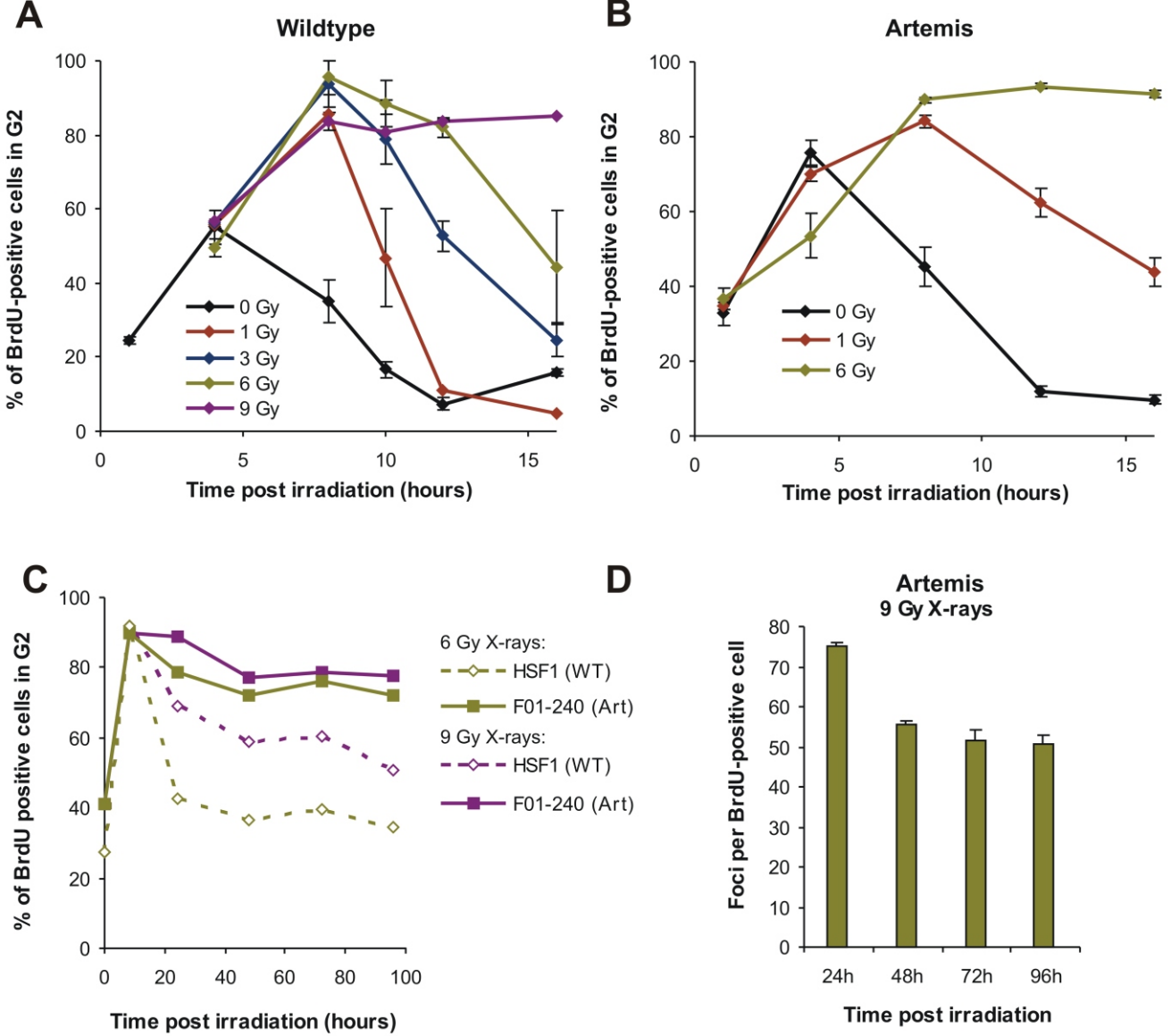
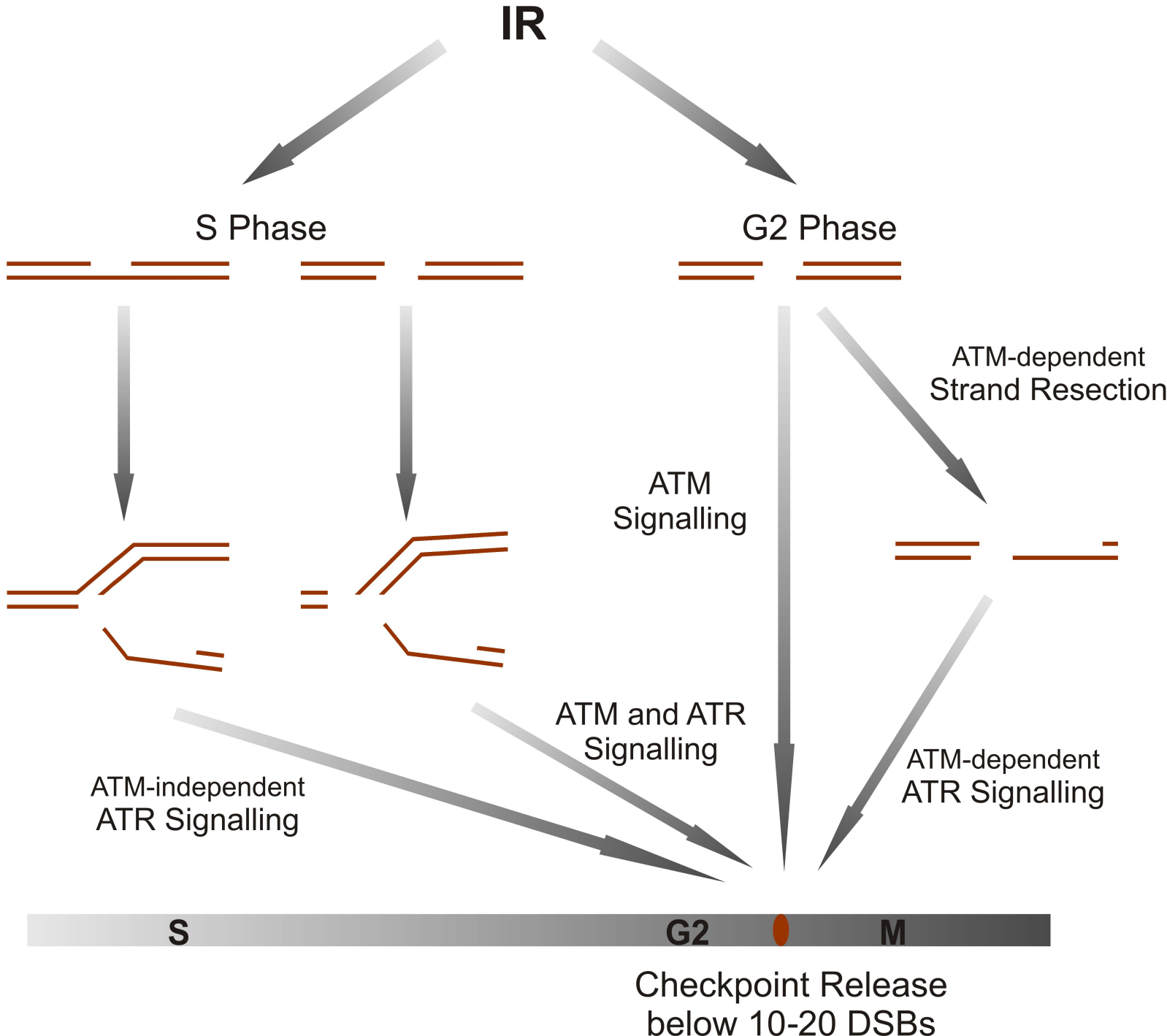
Time post labelling (hours)
– X-rays:
HSF1 (WT)
F01-240 (Art)
AT1BR (AT)
+ X-rays:
HSF1 (WT)
F01-240 (Art)
AT1BR (AT)
S
G2
G1
D
1 Gy X-rays in S
Foci per BrdU-positive G2 cell
30
25
20
15
10
5
0
8h
12h
Time post labelling
HSF1 (WT)
F01-240 (Art)
AT1BR (AT)


A
Wildtype
% of BrdU-positive cells in G2
Time post irradiation (hours)
0 Gy
1 Gy
3 Gy
6 Gy
9 Gy
B
Artemis
% of BrdU-positive cells in G2
Time post irradiation (hours)
0 Gy
1 Gy
6 Gy
C
% of BrdU positive cells in G2
Time post irradiation (hours)
6 Gy X-rays:
HSF1 (WT)
F01-240 (Art)
9 Gy X-rays:
HSF1 (WT)
F01-240 (Art)
D
Artemis
9 Gy X-rays
Foci per BrdU-positive cell
24h
48h
72h
96h
Time post irradiation


IR
S Phase
G2 Phase
ATM-dependent
Strand Resection
ATM
Signalling
ATM and ATR
Signalling
ATM-dependent
ATR Signalling
ATM-independent
ATR Signalling
S
G2
M
Checkpoint Release
below 10-20 DSBs